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LABORATORY MANUAL

Microbiology, Parasitology and Immunology

Chief Editor Liping Zhang

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Part 1

Basic Experiments

Experiment 1

Observation of Bacterial Morphology

▪ Objectives

(1) To learn how to use oil immersion objective lens to observe the morphology of bacteria.

(2) To understand the basic shapes and special structures of bacteria.

Ⅰ. Using Oil Immersion Lens of the Microscope

▪ Principle

Bacteria are relatively small in size, usually on the order of 1 μm in diameter. Bacteria can be visible only under light microscope employed with a 100-power objective lens with 10-power ocular lens.

Normally, when light waves travel from one medium into another, they bend. Therefore, as the light travels from the glass slide to the air, the light waves bend and are scattered similar to the "bent pencil" effect when a pencil is placed in a glass of water. The microscope magnifies this distortive effect. Also, if high magnification is to be used, more light is needed.

Immersion oil has the same refractive index (1.515) as glass (1.52), therefore, provides an optically homogeneous path between the slide and the lens of the objective. Light waves thus travel from the glass slide, into glass-like oil, into the glass lens without being scattered or distorting the image (Fig. 1-1). In other words, the immersion oil "traps" the light and prevents the distortion effect that is seen as a result of the bending of the light waves.

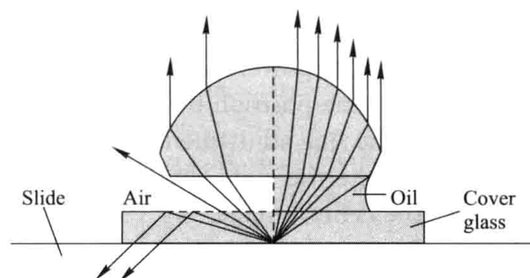

Fig. 1-1　The working principle of oil immersion objective lens

▪ Procedures

1. The identification of oil immersion objective lens(Fig. 1-2)

(1) The oil immersion objective lens is the longest.

(2) There are some marks on oil lens, such as objective magnification "100×", the numeric aperture "NA 1.25" and "Oil".

(3) Oil immersion objective lens has a black and white band.

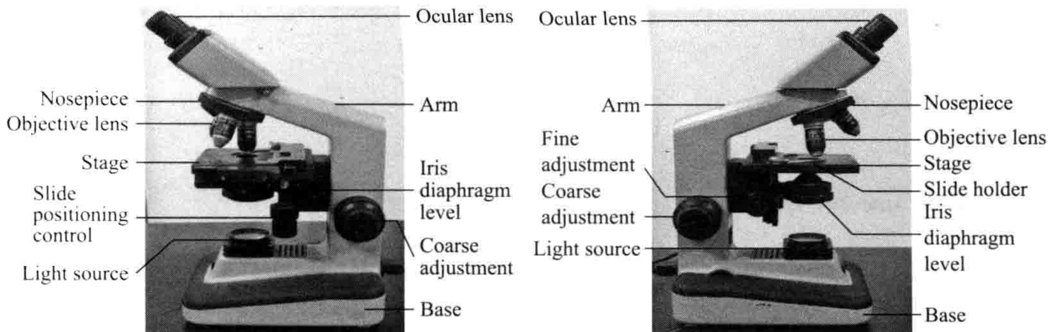

Fig. 1-2 An Olympus microscope

2. The procedure for using oil immersion objective lens

(1) Setting the focus

To switch on the power and open the aperture fully. Raise condenser to the same high level as the stage. Rotating the revolving nosepiece to make sure that the low power objective lens is clicked into place. Adjustment of light intensity may be necessary. The light should be strong when stained samples are observed, whereas the light should be weak when the unstained samples are observed.

(2) Observation of specimens using low-power objective lens

Lower the stage to its lowest position using the coarse focusing knob. Place the slide on the stage and hold it with a pair of stage clips. Locate desired portion of specimen in the center of the field. Rotate the coarse adjustment knob to raise the stage to the highest. While looking through the eyepiece, use the coarse-adjustment knob to lower the stage slowly to focus on the slide. After the outline of the specimens appear, turn the fine-adjustment knob until the object is in sharp focus. If there is no specimen visible, it is probably not in the field of view. Move the slide so that the specimen is directly over the condenser lens. Center the specimen in the field of view.

(3) Observation of specimens using high-power objective lens

Rotate the high-power objective into the position over the slide. While looking through the eyepieces, use only the fine adjustment knob to obtain a sharp image. Moving the stage clips to make the slide around until the area you wish to examine to be located at the central stage.

(4) Observation of slide using oil immersion objective lens

Rotate the objectives so that there is not a lens directly over the slide.

Apply a small drop of oil directly on the area to be observed. Slowly rotate the oil immersion objective lens into position over the slide. While looking through the eyepiece, USE ONLY the fine adjustment knob to focus on the specimen slide. Adjustment of light intensity by using the iris diaphragm may be necessary.

(5) Changing slides

To rotate out the oil immersion objective lens and then follow steps (2) through (4).

(6) Cleaning and maintenance

When you finish with your "scope" assignment, rotate the brightness knob to a minimum and switch off the power. Lower the stage to the lowest position using the coarse focusing knob. Make the ocular objective lens into place. Clean all lenses carefully with lens paper and place the low power objective in position, then replace the dust cover and return the microscope to the appropriate storage area. Wipe oil from the slide in one direction slightly.

▪ Note

The fine-adjustment knob can't be turned excessively to make sure the slide and objective lens not to be broken.

Ⅱ. Basic Morphology and Special Structure of Bacteria

▪ Materials and Procedures

To examine bacteria in 10 stained specimens with oil immersion objective.

1. The basic shapes of bacteria (Color Fig. 1-1)

(1) Cocci: *Staphylococci*, *Streptococci* and *Neisseria meningitidis*.

(2) Bacilli: *Escherichia coli*.

(3) Spiral bacteria: *Vibrio cholerae*.

Above specimens are Gram stained. Note the shapes, size, staining and arrangement of bacteria.

2. The special structures of bacteria (Fig. 1-3)

(1) Capsule

Streptococcus pneumoniae, Gram-positive. Look at it carefully for the location of capsules around bacteria.

(2) Flagella (Flagellum)

Salmonella typhi, Leifson stain. Pay attention to the morphology and distribution of flagella.

(3) Spore

Clostridium tetani, acid-fast stained. Observe carefully the size, dyeing and location of the spores in the bacterial cells.

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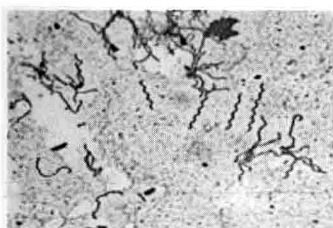
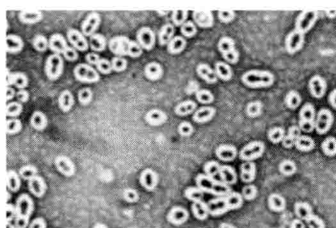
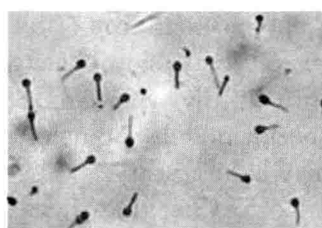


Flagella
Salmonella typhi



Capsule
Streptococcus pneumoniae



Spore
Clostridium tetani

Fig. 1-3 The special structures of bacteria

3. The characteristic morphology of bacteria

(1) Metachromatic granules

Corynebacterium diphtheria, Neisseria stained(to be improved). The bacterial cells are light blue with dark purple metachromatic granules on one end or both ends of the expansion.

(2) *Bacillus anthracis*

Gram stained. The rod-shaped bacteria are large with square ends and arranged in long bamboo-like chains. The spore shows an empty area, located in the central cell, and its diameter is less than the width of the cell.

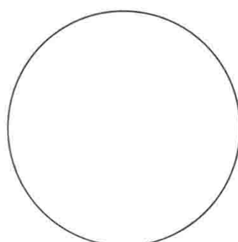
▪ Results

1. Draw following bacteria and fill in the blanks

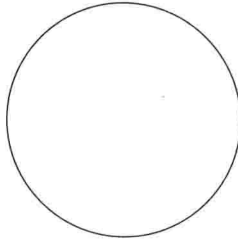
Bacteria	Drawing	Shape	Arrangement	Stained color
Staphylococci				
Streptococci				
Neisseria meningitidis				
Escherichia coli				
Vibrio cholerae				

2. Draw and label following bacterial special structures

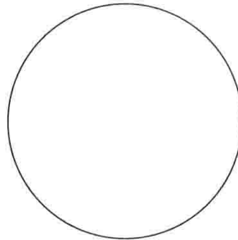
(1) Capsule



(2) Flagella



(3) Spore



QUESTIONS

1. How to identify the oil lens of a microscope?
2. Predict the consequences of the following:
   a. Using the coarse adjustment to focus the 100× objective.
   b. Do not clean the lenses before storage.
   c. Stage is not lowered before cleaning lens.
3. What organisms are not visible under your microscope?

(Liping Zhang 张力平)

Experiment 2

Bacterial Staining and Microscopy

- **Objectives**

(1) To learn how to perform Gram stain and acid-fast stain.
(2) To observe the results of Gram stain and acid-fast stain.

Ⅰ. Gram Stain

The Gram stain is the most widely used staining method in bacteriology. By this method, bacteria can be divided into two major categories: Gram-positive bacteria and Gram-negative bacteria. It is great significant to bacterial identification with potential evaluation of their pathogenicity and antibiotics sensitivity.

- **Principle**

The principle of Gram stain is not entirely clear. There are three main theories:

1. Permeability theory

Gram-positive cells hold thick peptidoglycan cell walls which are capable of retaining the crystal violet-iodine complex that occurs during staining, while the peptidoglycan layer of a Gram-negative cell is thin. Thus Gram-positive cells do not decolorize with ethanol, and Gram-negative cells do decolorize. This allows the Gram-negative cells to accept the counter stain safranin. Gram-positive cells will appear blue to purple, while Gram-negative cells will appear pink to red.

2. Isoelectric point theory

The isoelectric point of Gram positive cells is lower(pI=2 - 3)than that of Gram negative cells(pI=4 - 5). In the dye solution around pH 7.0, positive cells contain much more negative charge than negative cells. So they tightly bind to crystal violet with positive charge.

3. Chemical theory

Gram-positive cells contain a large amount of ribonucleic acid magnesium salts so that the complex of crystal violet and iodine binds to the cell

wall tightly and can not be extracted by 95% ethyl alcohol.

▪ Materials

(1) Organisms: Agar slant cultures of *Staphylococcus aureus* and *Escherichia coli*.

(2) Gram stain reagents: crystal violet, Gram iodine, 95% ethyl alcohol and safranin.

(3) Normal saline, glass slide, inoculating loop, alcohol lamp, bibulous paper, marking pencil.

▪ Procedures

1. Preparation of bacterial smears

(1) Marking

Divide the glass slide into two parts with a marker. Label the name of bacteria or serial number.

(2) Smearing

Place a small drop of physiological saline on the left side of your slide, one drop of saline on the right side. Flame your loop until the entire wire is red. Pick up the slant with your other hand, open the top, holding the cap with the little finger of the hand holding the loop. Flame the top of the tube. With the sterile loop, touch the bacterial growth (such as *Staphylococcus aureus*) on the slant. Flame off the tube and replace the cap. Smear the bacteria on the end of the loop into the drop of water on the slide (approximate 1 cm diameter). Repeat above steps for another slant culture (*Escherichia coli*).

(3) Air dry

Let the smear dry completely on a flat surface by room temperature. To speed up the drying, you can also heat the glass slide over the flame of the lamp but not too much.

(4) Fixation

While holding the glass slide at one end, pass the smear slowly through the flame of the lamp three times. This process effectively kills the bacteria, makes them stick to the slide by coagulation of their cellular proteins, and allows the dye to penetrate into the cells.

2. Staining

(1) Primary stain

Place glass slides on the staining shelves. Flood smear with crystal violet solution. Allow the crystal to stand for 1 minute. Pour off the stain. Gently rinse with tap water, remove excess water.

(2) Mordanting

Add iodine solution over the smear for 1 minute. Pour off the iodine solution. Gently rinse the smear with running water from one side of the glass slide to remove excess iodine.

(3) De-colorization

Add a few drops of 95% ethyl alcohol, hold the slide at an angle and allow the decolorizer to flow over the stained area of the slide, without it directly hitting the smear. The decolorizer should run clear for about 30 seconds until blue color stops coming out of the smear. Gently rinse with tap water.

(4) Counter staining

Stain the smear with safranin for 1 minute. Gently rinse the smear with tap water from one side of the glass slide to remove excess safranin.

3. Examination

Blot dry with bibulous paper and examine your specimen under oil-immersion objective. Note that morphology, arrangement and dyeing of bacteria.

▪ Results

Fill in the Table 2-1.

Table 2-1 The results of Gram stain

Bacteria	Color	The results of Gram stain
Staphylococci aureus		
Escherichia coli		

▪ Notes

(1) Add a drop of water on the slide, not too much.

(2) Only a very small amount of culture is needed.

(3) Spread the culture with an inoculation loop to an even thin film over a circle of 1.0 cm in diameter.

(4) Do not heat the slide for too long or keep it stationary over the flame.

(5) Timing carefully. Insufficient decolorization can make Gram-negative organisms falsely appear Gram-positive. Prolonged decolorization can cause Gram-positive bacteria to appear Gram-negative.

Ⅱ. Acid-fast Stain (Ziehl-Neelsen Method)

▪ Principle

The cell wall of *Mycobacteria* (e. g. *Mycobacterium tuberculosis*, *Mycobacterium leprae*) contains a large amount of fatty waxes (mycolic acids), which make the cells resist staining by ordinary methods. The cell walls are so durable that the staining process with carbol fuchsin must be carried out in heating conditions or with prolonged time. The bacteria that do not have

mycolic acids are easily decolorized with the acid-alcohol, while *Mycobacteria are not*. Other cells are then counterstained with methylene blue. So the acid-fast stain is used to differentiate *Mycobacteria* from non acid-fast bacteria.

▪ Materials

(1) Specimen: BCG (mimic sputum specimen of pulmonary tuberculosis patients).

(2) Stain reagents: Carbofuchsin, 3% acid alcohol, methylene blue.

(3) Glass slide, inoculating loop, alcohol lamp, filter paper.

▪ Procedures

1. Preparation of bacterial smears

Transfer a loop of BCG liquid from the bottom of bottle on a slide with a sterile inoculating loop to make a bacterial film. Proper smear preparation should produce a monolayer of organisms sufficiently dense for easy visualization. Air dry and fix as the procedures of Gram stain.

2. Stain

(1) Primary stain

Place the slide on the staining rack. Overlay carbofuchsin on the smears at room temperature for 10 minutes. Gently wash the slide with tap water and drain off the excessive water. Do it over flame when needed. After the formation of steam, maintain the staining for 5 minutes. When the slide is cooled, gently wash with water as above.

(2) Decolorization

Decolorize the smear with acid alcohol for about 1 minute until red color stops coming out of the smear. Gently wash with water as above.

(3) Counter staining

Flood methylene blue on the smears for 1 minute. and gently wash with water as above.

3. Observation

Drain the slide and blot dry with filter paper. Observe using oil immersion objective.

▪ Results

Fill in the Table 2-2.

Table 2-2 The results of acid-fast stain

Bacteria	Color	The result of acid-fast stain
BCG		
Non-acid-fast bacteria		

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